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Evolution of the *miR199-214* cluster and vertebrate skeletal development

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MicroRNA (miRs) are short non-coding RNAs that fine-tune the regulation of gene expression to coordinate a wide range of biological processes. MicroRNAs are transcribed from miR genes and primary miR transcripts are processed to approximately 22 nucleotide single strand mature forms that function as repressors of transcript translation when bound to the 3′UTR of protein coding transcripts in association with the RISC. Because of their role in the regulation of gene expression, miRs are essential players in development by acting on cell fate determination and progression toward cell differentiation. The *miR199* and *miR214* genes occupy an intronic cluster located on the opposite strand of the *Dynamin3* gene. These miRNAs play major roles in a broad variety of developmental processes and diseases, including skeletal development and several types of cancer. In the work reported here, we first deciphered the origin of the *miR199* and *miR214* families by following evolution of *miR* paralogs and their host *Dynamin* paralogs. We then examined the expression patterns of *miR199* and *miR214* in developing zebrafish embryos and demonstrated their regulation through a common primary transcript. Results suggest an evolutionarily conserved regulation across vertebrate lineages. Our expression study showed predominant expression patterns for both miR in tissues surrounding developing craniofacial skeletal elements consistent with expression data in mouse and human, thus indicating a conserved role of *miR199* and *miR214* in vertebrate skeletogenesis.

Keywords: microRNA, *miR199*, *miR214*, *dnm3*, *dnm3os*, zebrafish, skeletogenesis, miRNA evolution, vertebrates

Abbreviations: miR, microRNA; RISC, RNA-induced silencing complex; UTR, untranslated region; RNA, ribonucleic acid; VGD, vertebrate genome duplication; TGD, teleost genome duplication; RT-PCR, reverse transcription polymerase chain reaction; ISH, in situ hybridization; WISH, qhole mount in situ hybridization; hpf, hours post-fertilization

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Introduction

MicroRNAs (miRs) are short non-coding RNAs that help fine tune gene expression to coordinate a wide range of biological processes.[1,2] Primary transcripts of miR genes emerge from single or clustered genes and are sequentially processed to their final single strand form, which becomes active when loaded into the RNA-induced silencing complex (RISC).[3,4] Mature miRs generally repress protein expression by binding to specific sites on the 3′UTR of targeted transcripts. Binding involves perfect or near-perfect pairing of the miR seed region (nucleotides 2–9 of the approximately 22 nucleotide miR) to the 3′UTR of targeted transcripts and either induces transcript degradation or translation repression.[5,6]

Evidence suggests that miRs evolved from the RNAi machinery independently in several phyla of eukaryotes and experienced several rapid bursts of expansion within metazoans, especially among vertebrate lineages.[7] The increase of body plan complexity in early bilaterian, vertebrate, and mammalian evolution is correlated with the increasing number and diversification of microRNAs.[8-10] The origin and evolution of only a few miR families have been inferred,[11-14] but the evolutionary mechanisms underlying the emergence of novel miRs, duplication of existing miRs, and retention or loss of miR duplicates remain unclear.

Several miRs play critical regulatory roles at various steps of bone formation. They regulate cell differentiation and/or proliferation by modulating the activity of crucial skeletal transcription factors.[15-21] Various miRs are players in bone development and bone mineralization

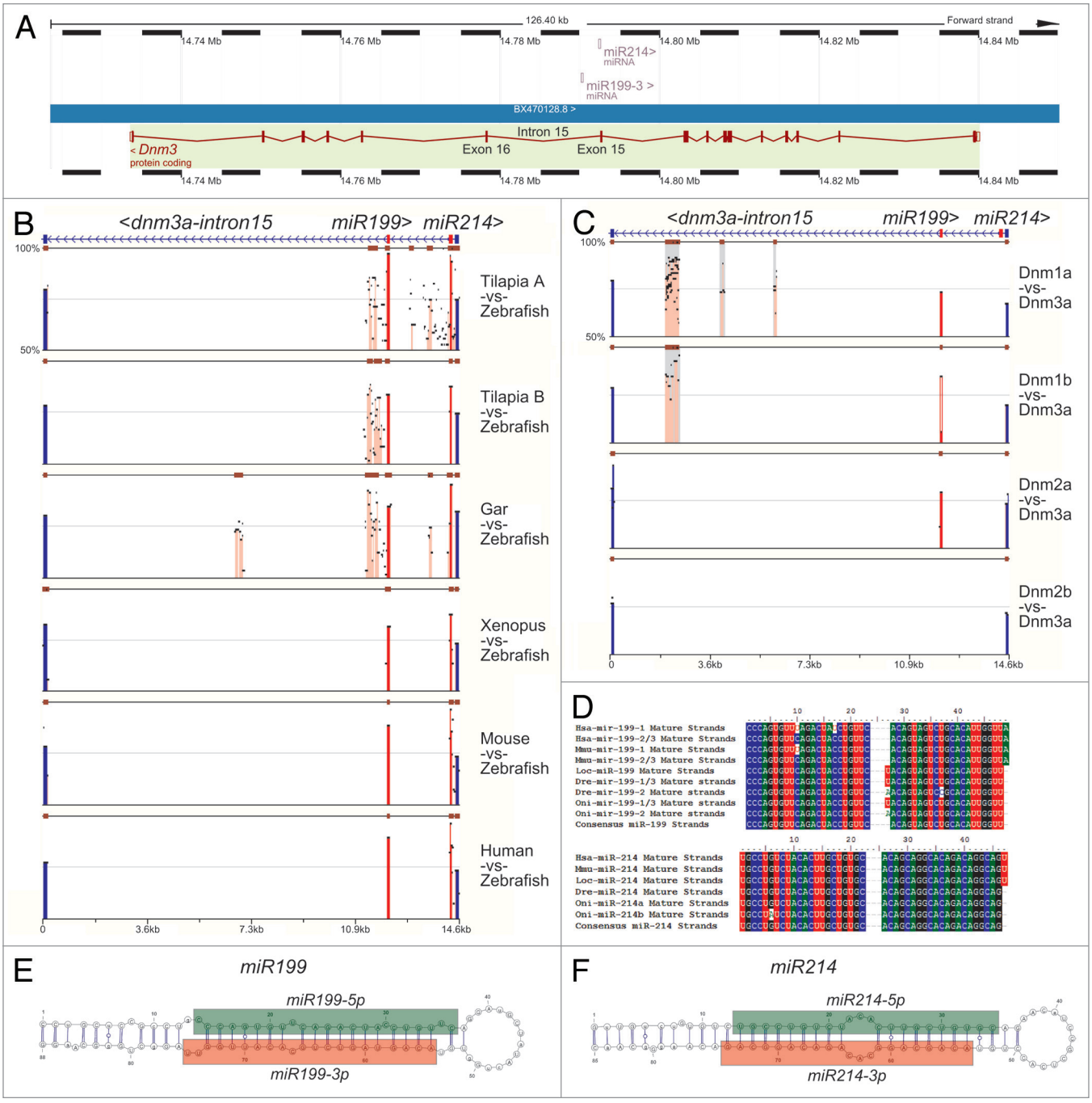

Figure 1. Genomic organization and conservation of *miR199* and *miR214* genes across vertebrates. (**A**) *miR199* and *miR214* reside in an intronic cluster opposite to the coding strand of *Dnm3* in zebrafish (modified from Ensembl[84]). (**B**) Graphical output of zPicture alignment of intron 15 of various vertebrate *Dnm3* genes to intron 15 of zebrafish *Dnm3*. The ends of exons 15 and 16 of *dnm3* are represented in blue, miRs in red, and other conserved non-coding elements in pink. The baseline is set to 50% identity and the intermediate line represents 75% identity. (**C**) Graphical output of zPicture alignment of intron 15 of all zebrafish *Dnm* genes on zebrafish *Dnm3a*. (**D**) Alignment of mature sequences of vertebrate *miR199* and *miR214* genes. The three columns containing all gaps were inserted to separate the two mature sequences visually. The consensus hairpin structure of *miR199* (**E**) and *miR214* (**F**) were predicted by alignment of several primary-miR sequences across the vertebrate lineage (see also **Fig. S4**). Consensus mature sequences are highlighted on each hairpin.

diseases, suggesting that they participate not only in skeleton formation, but also in maintaining the skeleton in a healthy state.[22-24]

miR199 and *miR214* play roles in the differentiation of mammalian skeletal precursor cells into osteoblasts or chondrocytes.[25-31] In addition, these miRs function in the development of muscle and heart,[32-35] and regulate the development and progression of various cancers.[36-40]

miR199 was first identified in human osteoblast sarcoma cells[41] and mouse embryonic stem cells.[42] *miR214* was first identified by sequence homology between human and mouse[43] and its expression was further validated in mouse.[44] The *miR199* and *miR214* genes are genomic neighbors and are expressed from a common transcript in mouse and human.[25,27] Homologs of *miR199* and *miR214* have now been identified computationally in more than 20 vertebrate species, but paralogous and orthologous relationships and the evolutionary origin and subsequent history of these homologs have not yet been investigated. Here we decipher the origin and evolution of both miR families, investigate the evolutionarily conserved role of *miR199-214* for skeletogenesis, and propose a new and harmonized gene nomenclature for *Dynamin*, *miR199*, and *miR214* genes across vertebrates.

Results

miR199 and *miR214* form a vertebrate-specific conserved cluster within the *Dnm3* gene

Our search for *miR199* and *miR214* gene sequences in zebrafish led to the identification of an evolutionary conserved *miR199-214* cluster on the opposite strand of a *dynamin3* (*dnm3*) gene, an arrangement that is conserved in all vertebrates examined (**Fig. 1A and B**), including mammals.[25] In zebrafish, the cluster is located on the opposite strand of *dnm3*-intron15 as depicted in **Figure 1A**. The conserved location in orthologous introns of orthologous genes among vertebrates was verified by aligning the orthologous *Dnm3* intron of various vertebrates to zebrafish *dnm3*-intron15, where exons (the ends of exon15 and exon16 shown in blue in both sides of **Fig. 1B**), and both *miR199* and *miR214* (depicted in red in **Fig. 1B**), show a high degree of identity among all vertebrate sequences examined from tilapia to human. Several conserved non-coding elements, possibly regulatory elements or processing sites, were also detected in this intron (pink bars in **Fig. 1B and C**). The distance separating *miR199* and *miR214* in the genomes of studied species varied from 1207 to 5750 nt (**Table S1**) suggesting the existence of a conserved polycistronic transcript called *Dnm3os* (opposite strand), which has been identified in both human and mouse genomes.[25,27] Vertebrates contain several *Dnm* genes, with tetrapods having three (*Dnm1, Dnm2,* and *Dnm3*) and teleosts often having six genes (*dnm1a, dnm1b, dnm2a, dnm2b, dnm3a,* and *dnm3b*), duplicate pairs that arose in the teleost genome duplication.[45-47] Vertebrates also have several paralogous copies of *miR199* that are already annotated and reside in paralogous introns of *Dynamin* paralogs; **Figure 1C** shows the situation for the five zebrafish *dnm* paralogs, which harbor conserved *miR199* genes in paralogous introns of four *dnm* genes; only *dnm2b* lacks a *miR199* paralog.

Table 1. The location of miRs within each *Dynamin* cluster in vertebrates.

Locus		1			2		3		
Gene		*Dnm1*	*miR199-1*	*miR3154*	*Dnm2*	*miR199-2*	*Dnm3*	*miR199-3*	*miR214*
Homo sapiens		●	●	●	●	●	●	●	●
Mus musculus		●	●	●	●	●	●	●	●
Gallus gallus		●	●		●		●	●	●
Xenopus tropicalis		●			●	●	●	●	●
Latimeria chalumnae		●	●		●	●	●	●	●
Lepisosteus oculeatus		○	○		○	○	○	○	○
Danio rerio	A Locus	●	●		●	●	●	●	●
Gadus morhua	A Locus	●	●		●	●			
Takifugu rubripes	A Locus	●	●		●	●	●	●	●
Oryzias latipes	A Locus	●	●		●	●	●	●	●
Gasterosteus aculeatus	A Locus	●	●		●	●			
Tetraodon nigroviridis	A Locus	●	●		●	●	●		●
Oreochromis niloticus	A Locus	●	●		●	●	●	●	●
Xiphophorus maculatus	A Locus	●	●		●	●	●	●	●
Danio rerio	B Locus	●	●		●				
Gadus morhua	B Locus	●			●		●		●
Takifugu rubripes	B Locus	●			●		●	●	●
Oryzias latipes	B Locus	●			●		●	●	●
Gasterosteus aculeatus	B Locus	●			●		●	●	●
Tetraodon nigroviridis	B Locus	●			●		●	●	●
Oreochromis niloticus	B Locus	●			●		●	●	●
Xiphophorus maculatus	B Locus	●			●		●	●	●

Plain black discs, plain gray discs, and empty circles represent annotated miRs, miRs that are predicted in Ensembl but un-annotated, and miRs that are newly identified in this work, respectively.

In addition to *miR199*, *Dnm3* in all non-teleost vertebrate genomes yet examined has a single copy of *miR214* associated with *miR199.* In contrast, several teleosts possess a duplicated copy of *miR214*, one in each of their two *dnm3* paralogs (**Fig. 1B**). In zebrafish, *miR214* is present in *dnm3*, but no other *dnm* paralog shows any similarity trace to the *miR214* gene (**Fig. 1C**). In addition to the conserved location of *miR199* and *miR214* among vertebrate genomes, their mature sequences and hairpin structures are also preserved across paralogs and orthologs (**Fig. 1D–F**). The conserved association of *Dynamin* genes and intronic *miR199* and *miR214* genes suggests that the protein-coding gene and the microRNA genes embedded within it share an evolutionary history.

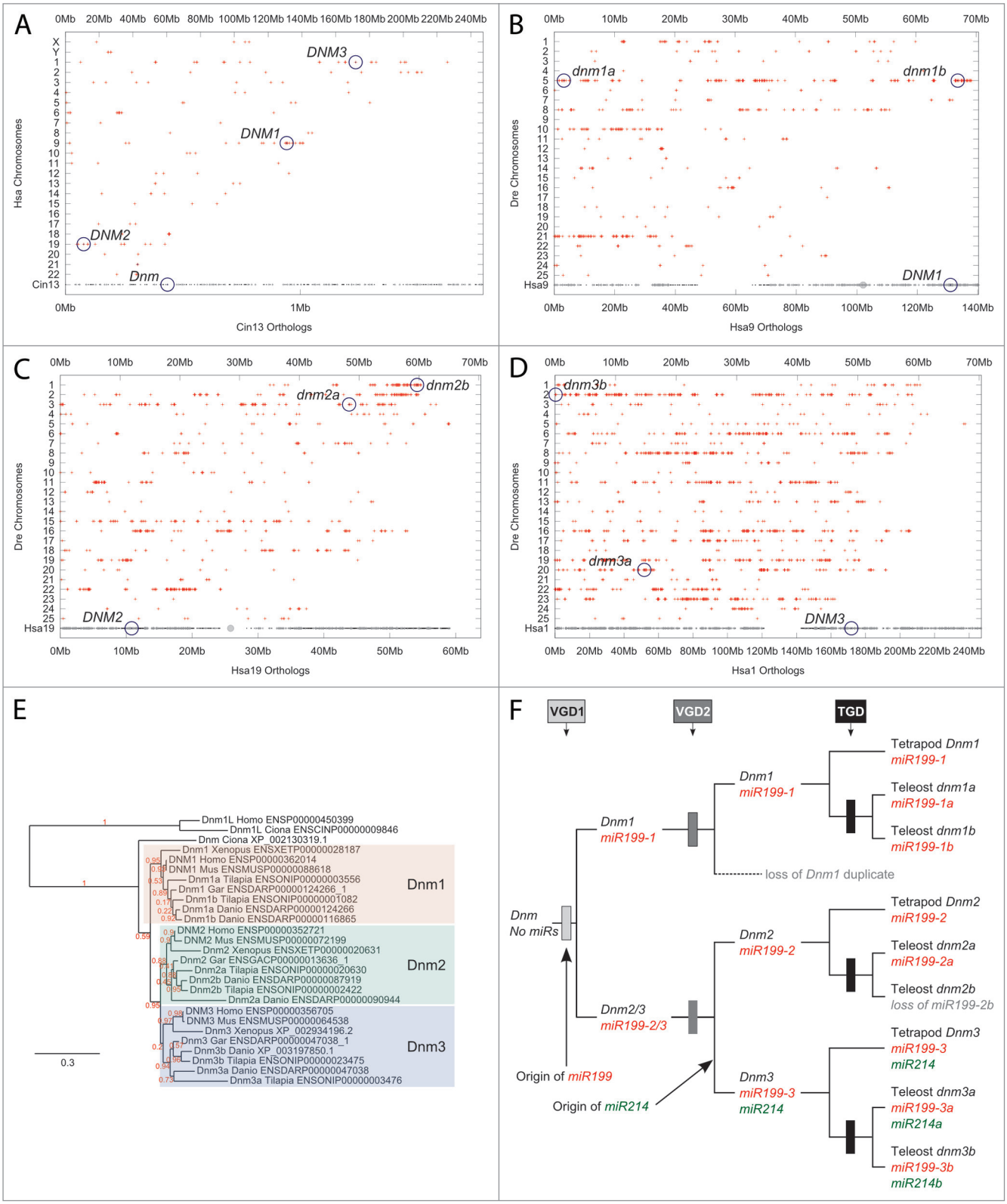

Figure 2. Evolution of *Dynamin* loci. (**A**) Orthology dot plot of *Ciona intestinalis* chromosome 13 on the human genome showing orthology relationships of the single Ciona *Dnm* gene with the three human *Dnm* paralogs on chromosomes 1, 9, and 19, which are known paralogons.[50] Human chromosomes are shown with the location of the human orthologs of Ciona genes plotted proportional to their location across chromosomes scaled in size relative to other human chromosomes. (**B–D**) Orthology dot plot of Human chromosomes containing *Dynamin* genes on the zebrafish genome, showing a one-to-two othology relationship between human and zebrafish *Dnm* genes. (**E**) Phylogenetic tree of the Dynamin family. (**F**) The most parsimonious scenario depicting putative *Dynamin* locus evolutionary history in the vertebrate radiation. VGD1, VGD2, and TGD, respectively indicate the first and second rounds of vertebrate whole genome duplication and the teleost genome duplication events.

Table 2. Name correspondence between the previous alias and the newly proposed nomenclature.

Protein coding Locus	*Dnm1*	*Dnm2*	*Dnm3*	
miR gene	*miR199-1*	*miR199-2*	*miR199-3*	*miR214*
Homo sapiens	*MIR199B*	*MIR199A1*	*MIR199A2*	*MIR214*
Mus musculus	*MiR199b*	*Mir199a-1*	*Mir199a-2*	*Mir214*
Gallus gallus	*mir-199-1*		*mir-199-2*	*mir-214*
Xenopus tropicalis		*miR-199b*	*miR-199a*	
Latimeria chalumnae				
Lepisosteus oculeatus				

Protein coding Locus	*Dnm1a*	*Dnm1b*	*Dnm2a*	*Dnm2b*	*Dnm3a*		*Dnm3b*	
miR gene	*miR199-1a*	*miR199-1b*	*miR199-2a*	*miR199-2b*	*miR199-3a*	*miR214a*	*miR199-3b*	*miR214b*
Danio rerio	*mir199-2*	*CU929237.1*	*mir199-3*		*mir199-1*	*mir214*		
Gadus morhua								
Takifugu rubripes	*mir-199-2*		*mir-199-3*		*mir-199-1*	*mir-214*		
Oryzias latipes	*mir-199a-3*		*mir-199a-2*		*mir-199a-1*		*mir-199a-4*	*mir-214*
Gasterosteus aculeatus								
Tetraodon nigroviridis	*mir-199-2*		*mir-199-3*			*mir-214*	*mir-199-1*	
Oreochromis niloticus								
Xiphophorus maculatus								

Searches of genome databases revealed no *miR199* or *miR214* motifs outside the vertebrate lineage. Because among vertebrates, both *miR199* and *miR214* were always located within an intron of a *Dynamin* paralog, we looked for miR genes within *Dynamin* genes in non-vertebrate metazoans, ranging from the placozoan *Trichoplax adhaerens* to the urochordate *Ciona intestinalis*. These searches revealed a single copy of *Dnm* in each species, confirming previous observations.[48] Although miRBase has an entry for a *miR199* gene in the urochordate *Ciona intestinalis* (MI0007174), neither the proposed 3p mature sequence nor the stem-loop have matches to the latest genome assemblies of either *C. intestinalis* or *C. savigny* (KH and CSAV2.0, respectively). Because the urochordate *Oikopleura dioica* also lacks this *miR199* gene,[49] we consider the *miR199* annotation for *C.savigny* to be in error. We identified three conserved *Dynamin* loci in non-teleost gnathostomes, and five to six loci in teleosts. An agnathan, the lamprey *Petromyzon marinus*, has one annotated *miR199* gene found in the genome assembly in a putative intron of an incompletely annotated *Dynamin* gene (ENSPMAG00000006300 on Scaffold GL476397), but the lamprey *Dnm* gene is incompletely annotated on its 3' end and a conserved intronic location for the lamprey *miR199* gene cannot be ruled out. We find no evidence for a lamprey *miR214* gene. The little skate *Leucoraja erinacea*, a cartilaginous fish, appears to possess a single copy of *miR214* located on Contig 65552. No clear evidence of a *Dnm* gene or a *miR199* gene was found in the skate genome assembly (Build 2), but because the skate genome was sequenced at low coverage, missing loci could be due to missing data rather than missing genes. The absence of *miR199* and *miR214* genes in non-vertebrate genomes and their clear presence in all vertebrate species with well-assembled genomes demonstrate that both miR families are vertebrate-specific, agreeing with previous observations.[9] Moreover, the presence of *miR199* in the lamprey genome suggests that this *miR* gene arose around or at the onset of the vertebrate radiation. The presence of *miR214* in the little skate genome suggests that *miR214* emerged between the onset of the vertebrate radiation and the divergence of gnathostome fish.

The evolution of *miR199* and *miR214* in vertebrates

To decipher evolutionary relationships between *miR199* and *miR214* homologs, we studied the global evolution of the *Dynamin* gene family and its miR gene inhabitants. In general, all non-teleost vertebrate genomes possess three *Dynamin* paralogs, *Dnm1, Dnm2,* and *Dnm3*, each containing a single copy of *miR199* on the opposite strand of a paralogous intron. Only *Dnm3* possesses a *miR214* sequence, which forms a cluster with *miR199* (**Table 1**, **Fig. 1A–C**). *Dnm2* in the current assemblies of the genomes of chicken and other birds (the duck *Anas platyrhynchos*, the flycatcher *Ficedula albicollis*, and the Zebra Finch *Taeniopygia guttata*) lack *miR199*, likely because *Dnm2* is incompletely assembled and poorly annotated in these reference genomes, and because the anole lizard *Anolis carolinensis* and the Chinese soft-shell turtle *Pelodiscus sinensis* both display a *miR199* gene at the expected intronic location. On the other hand, the lack of *miR199* in *Xenopus Dnm1* probably reflects species-specific loss because *Dnm1* is well assembled in this genome and all sequenced sauropsids possess a *miR199* gene within an intron of *Dnm1*.

To learn whether vertebrate *Dnm* genes originated in two rounds of vertebrate genome duplication (the VGD1 and VGD2 events[50]), we constructed chromosome-wide orthology plots using the Synteny Database.[51] These analyses revealed co-orthology of the unique Ciona *Dnm* locus located on chromosome Cin13 with the three human *Dynamin* loci located on chromosomes Hsa1, Hsa9, and Hsa19 (**Fig. 2A**). Likewise, a one-to-one relationship of conserved synteny was evident between the *C. intestinalis Dnm* genomic region and all three human *Dynamin* genes (**Fig. 2A**; **Fig. S3**). Because the human *DNM2* and *DNM3* genomic regions share a number of gene losses compared with *DNM1* and the *C. intestinalis Dnm* region, by parsimony, *DNM2* and *DNM3* were likely duplicated in the VGD2 event, while *DNM1* and the *DNM2/3* genes likely arose in the VGD1 event, with the sibling of *DNM1* having gone missing from all extant vertebrates (**Fig. S3**). This finding also reinforces the vertebrate-specific origin of the

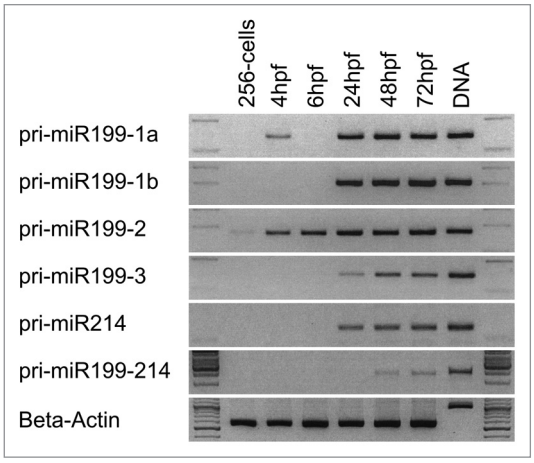

Figure 3. RT-PCR analysis of *miR199*, *miR214*, and *miR199-214* transcript expression during zebrafish early development. Expression was studied at the 256-cell stage, and at 4, 6, 24, 48, and 72 hpf. Genomic DNA was loaded on last lane as size control. *b-actin* was used as internal control for genomic contamination of the samples due to intron possession.

miR199 and *miR214* families because the *Ciona* Chromosome 13 region containing *Dnm* is clearly orthologous to the *DNM*-containing regions of the human genome, but these miRs are not present in the *Ciona Dnm* gene or anywhere else in the genome of this non-vertebrate chordate or any other non-vertebrate metazoan.

Three additional miRs appear within some mammalian *Dynamin* paralogs (**Table 1**; **Table S1**). The protein-coding strand of the human *DNM2* gene (i.e., the opposite strand from the coding strand for *miR199-2*) contains *MIR638* and *MIR4748* in intron 1 and 5, respectively. These two miRs were found only in primates (*Callithrix jacchus*, *Macaca mulatta*, *Gorilla gorilla*, and *Homo sapiens*) and not in mouse or other non-primate vertebrates, and are thus primate-specific miRs. *MIR3154*, located near *miR199-1* in *Dnm1*, occurs in most available eutherian genomes (*Loxodonta africana*, *Procavia capensis*, *Sus scrofa*, *Rattus norvegicus*, *Mus musculus*, *Homo sapiens*) but not in non-eutherian vertebrates, including marsupial and protherian mammals (the opossum *Monodelphis domestica* and the platypus *Ornithorhynchus anatinus*), indicating that it is a eutherian-specific miR. *MIR3154* is of particular interest because it is located in intron 15, on the same strand and less than 120 nt upstream of *miR199-1*, which strongly suggests the expression of these two miRNAs in a common pri-miR transcript. In addition to miR emergence, a new miR, *miR3120*, was revealed by high-throughput sequencing in human, rat, cow and in the Jamaican fruit bat[52] and the *miR3120* gene overlaps *miR214* with nearly complete overlap but on the opposite strand, and is thus categorized as a mirror-miR.[53,54] Expression of mirror-miRs has been demonstrated in *Drosophila*,[53] but to our knowledge, *miR3120* is the only experimentally documented mirror-miR in vertebrates.[54] As one would expect from an intronic miR, *miR3120* has been shown to be co-expressed with and to regulate important aspects of cellular function similar to its same-stranded host coding gene *Dnm3*.[54]

In teleost fish, five to six *dynamin* genes have already been annotated (**Table 1**; **Table S1**). Orthology plots between human and zebrafish *dynamin* loci show that each human *DNM* gene has two co-orthologs in zebrafish (**Fig. 2B–D**), indicating that they are most likely the result of the teleost genome duplication (TGD). The lack of a duplicated *dnm3* gene in zebrafish, cod and stickleback is probably due to mis-assembly of the genome sequence because partial copies of additional *dnm3* genes can be identified by protein blast on un-annotated regions at extreme ends of chromosomes in both zebrafish and stickleback (**Table S1**), and the cod assembly remains incomplete for this gene. For zebrafish, the incomplete *dnm3b* gene that we found near the left telomere of chromosome 2 was used as the location of the duplicated *dnm3* gene as shown on **Figure 2D**. Among those five or six *dynamin* loci in teleosts, some—such as *dnm2b* in zebrafish—do not display any conserved miR sequences (**Fig. 1C**). In some cases, the apparent lack of a *miR199* or *miR214* paralog is likely the result of assembly problems (gaps, incomplete gene assemblies, contig junctions, and low genome coverage). **Table S1** presents information on possible mis-assembly problems. The only teleost gene losses we can point to with confidence are the loss of *miR199-1b* from *dnm1b* in tetraodon and the lack of a *miR199* paralog from *dnm2b* in the platyfish *Xiphophorus maculatus* and some other teleosts (**Table S1**).

An examination of *miR199* or *miR214* mature sequence evolution shows how highly conserved sequences within and among species (**Fig. 1D**; **Figs. S4 and 5**). Based on Stockholm alignments of predicted hairpin sequences for several *miR199* and *miR214* genes, a highly conserved hairpin structure exists for both miRs across vertebrates (**Fig. 1E and F**; **Fig. S4**). Indeed, across all vertebrates *miR199-5p* sequences are identical, except that mouse and human *miR199-1-5p* show one and two base differences, respectively, outside of the seed region (**Fig. 1D**). It is noteworthy that the C-to-U modification observed in mouse *miR199-1-5p* is also shared by human *miR199-1-5p*, and was also found in all mammals from the opossum *Monodelphis domestica* to human, but not in *Xenopus tropicalis* or the lizard *Anolis carolinensis*. This finding suggests that this C-to-U mutation occurred in the stem mammalian lineage.

The *miR199-3p* sequence is also highly conserved throughout vertebrates with only one or two base differences outside of the seed region in tilapia and zebrafish *miR199-2*, respectively. The single nucleotide offset between the annotated sequences of tetrapod and actinopterygian *miR199-3p* might not reflect the reality of the mature sequences (**Fig. 1D**). Indeed, both mouse and human possess a U nucleotide just before the 3p strand in the hairpin and all actinopterygians possess an A nucleotide at the end of the same strand at the same location in their hairpin (**Fig. S5**). The difference in mature sequence position along

the pre-miR sequences could be due either to real biological modification of miRNA processing or to a problem in the mature sequence deposited in miRBase. Deeper micro-RNA sequencing experiments in these species will eventually resolve this issue.

Likewise, *miR214-5p* and *3p* strands are perfectly conserved in vertebrates except for a G-to-A nucleotide change in the tilapia *miR214b* duplicate (**Fig. 1D**). In contrast to *miR199*, the one-nucleotide difference at the 3p end of the *miR214-3p* strand is unlikely due to mislabeling of the mature sequence because actinopterygians possess a two-nucleotide indel rather than a UC at that position in the hairpin sequence (**Fig. S5**). This high degree of sequence conservation among paralogs within species and among orthologs between species suggests that the roles of paralogs and orthologs may be evolutionarily and functionally conserved in the development or activity of vertebrate-specific features.

Because mature *miR199* sequences are evolutionarily conserved, sequence analyses are insufficient to infer phylogenetic relationships among *miR199* paralogs. Thus, to elucidate phylogenetic relationships among paralogous *miR199* loci, we retraced the evolution of the Dynamin host protein in the vertebrate radiation using the *C.intestinalis* Dnm protein as an outgroup to vertebrates and the closest related protein to the true Dynamin family, Dynamin-Like 1 (DNM-1L) from Ciona and human to root the tree (**Fig. 2E**). Results show that first the Ciona Dnm protein branched basal to all vertebrate Dynamin proteins, thus confirming the ancestral state of this unique non-vertebrate metazoan protein. Second, the sequences of proteins judged to be paralogs from conserved synteny analysis tended to form monophyletic clusters, confirming the one-to-one or one-to-two orthology relationship among vertebrates. Finally, the phylogenetic tree displayed Dnm1 sequences as the sister group to a clade containing both Dnm2 and Dnm3 proteins, bolstering the conclusion from conserved syntenies that suggested a duplication of the ancestral *Dnm* gene to form the *Dnm1* and *Dnm2/3* gene following VGD1, and the origin of *Dnm2* and *Dnm3* after VGD2. These observations agree with

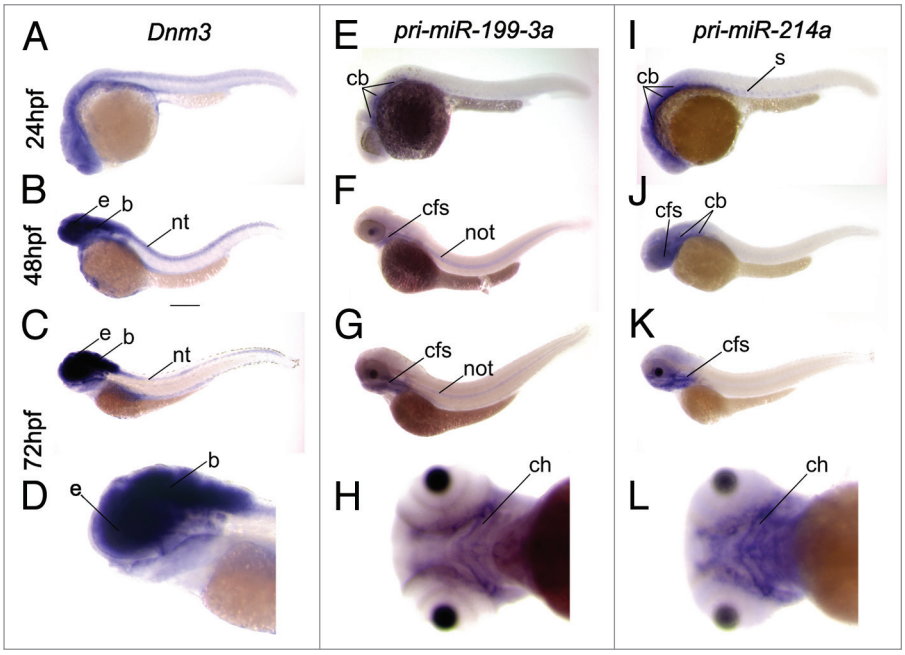

Figure 4. Whole mount in situ hybridization (WISH) of *dnm3* (**A–D**), *pri-mir199-3* (**E–H**) and *pri-miR214* (**I–L**) during zebrafish early development at 24 hpf (**A, E, and I**), 48 hpf (**B, F, and J**), and 72 hpf (**C, D, G, H, K, and L**) in lateral view (**A–G, I–K**), and ventral view (**H and L**). b, brain; cb, ceratobranchial; cfs, craniofacial skeleton; ch, ceratohyal; e, eyes; not, notochord; nt, neural tube; s, somites.

and extend the previously incomplete phylogeny of the *Dynamin* gene family.[48,55] We could find neither the VGD2 duplicate of *Dnm1* nor any trace of its locus remnant in any available sequenced genome, leading to the conclusion that this duplicate was lost at or soon after VGD2.

In summary, these data allow us to construct an evolutionary scenario for the origin of DNM-related miRs (**Fig. 2F**). First, the model shows the non-vertebrate metazoan *Dynamin* locus is related to the three mammalian *Dnm* loci by VGD1 and VGD2, and then to the six teleost *Dynamin* loci by the TGD. Both conserved synteny and phylogenetic analysis suggest that VGD1 gave rise to two genes, *Dnm1* and *Dnm2/3*, and that VGD2 produced *Dnm1* and its duplicated that is now lost, as well as *Dnm2* and *Dnm3*, both of which have been retained. During the TGD, all three vertebrate loci were further duplicated in teleosts. The presence of a *miR199* gene in the lamprey genome supports the emergence of *miR199* early in the vertebrate radiation, before the divergence of the agnathan and gnathostome lineages. On the other hand, the absence of *miR214* in lamprey, which cannot be verified with certainty due to genome assembly issues, and the presence of *miR214* in the little skate genome suggest the emergence of *miR214* between the onset of the vertebrate lineage and the divergence of bony vertebrates from cartilaginous fish. Parsimony favors the emergence of *miR214* directly within *Dnm3* gene after VGD2. The other possible scenario, hypothesizing the emergence of *miR214* before the divergence of jawed and jawless fish would imply the loss of a *miR214* duplicate within *Dnm2*, which is less parsimonious.

This new understanding of the origins of *miR199* and *miR214* gene families compels a new and harmonized gene nomenclature for *Dynamin*, *miR199*, and *miR214* genes across vertebrates; **Table 2** shows this nomenclature system. This system starts with a coherent nomenclature for *Dynamin* genes based on their origin and modeled on the human nomenclature which drives the naming of the miR genes based on their *Dynamin* host gene names. The system has the advantage that it harmonizes names across species from an evolutionary perspective and includes relationships between miR genes and *Dnm* genes.

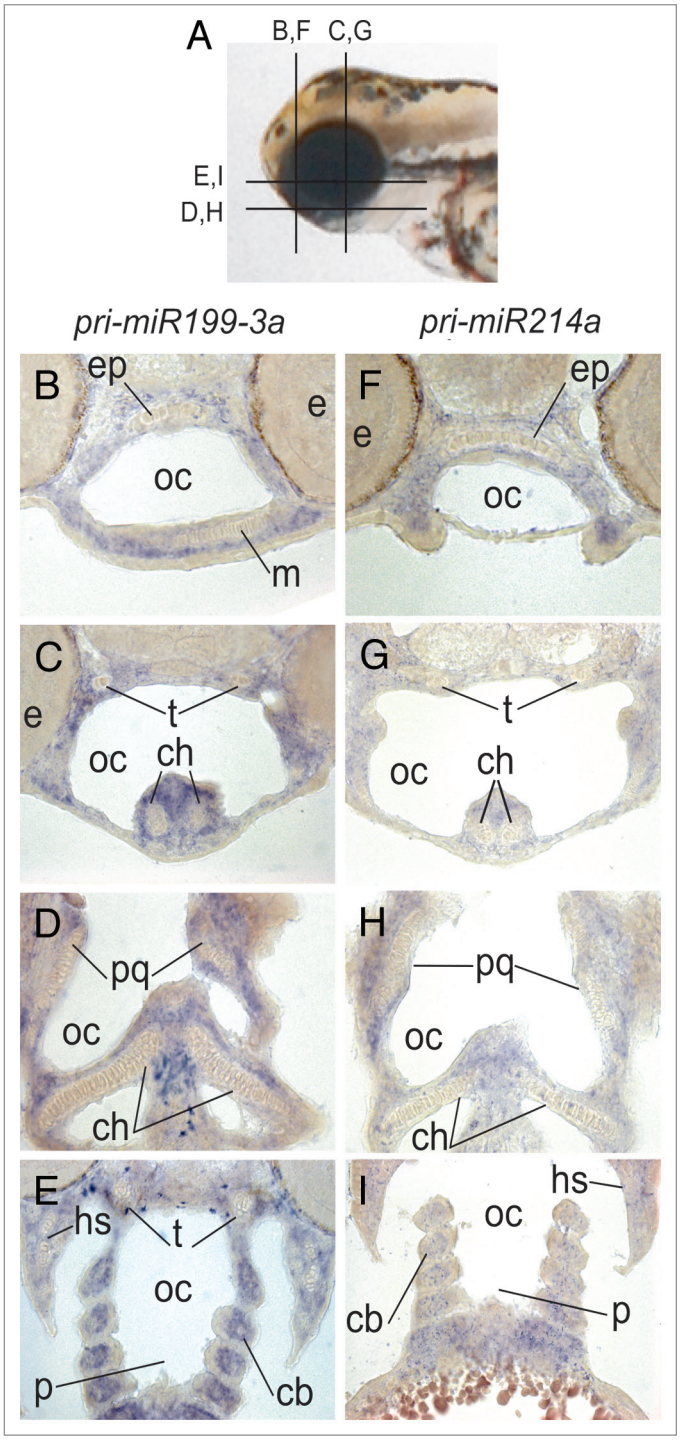

Figure 5. In situ hybridization of *pri-miR199-3* and *pri-miR214* on transverse (**B, C, F, and G**) and coronal cryosections (**D, E, H, and I**). Locations of sections are shown in (**A**). cb, ceratobranchial; ch, ceratohyal; e, eye; ep, ethmoid plate; hs, hyosymplectic; m, meckal's; oc, oral cavity; p, pharynx; pq, palatoquadrate; t, trabecula.

***miR199* and *miR214* are partially co-regulated**

To understand the evolution of *miR199-214* cluster functions, we studied the expression of primary miR transcripts during early zebrafish development by RT-PCR (**Fig. 3**). Results showed that *pri-miR199-1b*, *pri-miR199-3*, and *pri-miR214* displayed similar temporal expression patterns with no expression detected before 24hpf, after which transcripts were detected and persisted until at least 72 hpf. Note that *pri-miR199-3* and *pri-miR214* displayed similar temporal expression patterns during development, suggesting common regulation. Intriguingly, in biological replicates, *miR199-1a* displayed a distinct expression pattern in which transcripts were detected at 4 hpf, not at 6 hpf, and then again between 24 hpf to 72 hpf, thus showing a transient expression at the onset of genome activation.[56] This expression pattern is puzzling because the mature sequences produced by *miR199-1a* are identical to the ones generated by *miR199-1b* and *miR-199-3*. The *pri-miR199-2* gene displayed a different temporal expression pattern; its transcripts were faintly detected as early as the 256-cell embryo (**Fig. 3**), demonstrating maternal inheritance because the embryonic genome is still inactive at that developmental stage.[56] From 4 hpf (the onset of the mid-blastula transition and zygotic transcription in zebrafish[57]) to 72 hpf, the expression level of *pri-miR199-2* was much higher than at 256-cell stage (**Fig. 3**) demonstrating early zygotic expression of *pri-miR199-2* as soon as the zygotic genome activates. Because *pri-miR199-2* has a different mature sequence on the 3p strand than all other zebrafish *pri-miR199s* (**Fig. 1D**), it may have different mRNA targets and hence distinct roles in development.

To determine whether *miR199-3* and *miR214* are transcribed in a common transcript, we designed primers that flank both miR genes and amplify a fragment about 2350 nt long. Consistent with the genomic arrangement of the *miR199-214* cluster, RT-PCR experiments using these primers detected a common *pri-miR199-214* transcript at low levels, between 48 and 72 hpf (**Fig. 3**). This result demonstrates the existence of a conserved *pri-miR199-214* transcript in zebrafish as occurs in mammals.[27,28] Furthermore, the presence of a common transcript demonstrates common transcriptional regulation of both miRs in the *miR199-214* cluster and suggests an evolutionarily conserved cooperative role of the cluster among vertebrates.

To analyze the spatial component of *dnm3*, *miR199-3* and *miR214* expression, we designed pri-miR probes for in situ hybridization.[58] These probes extended for

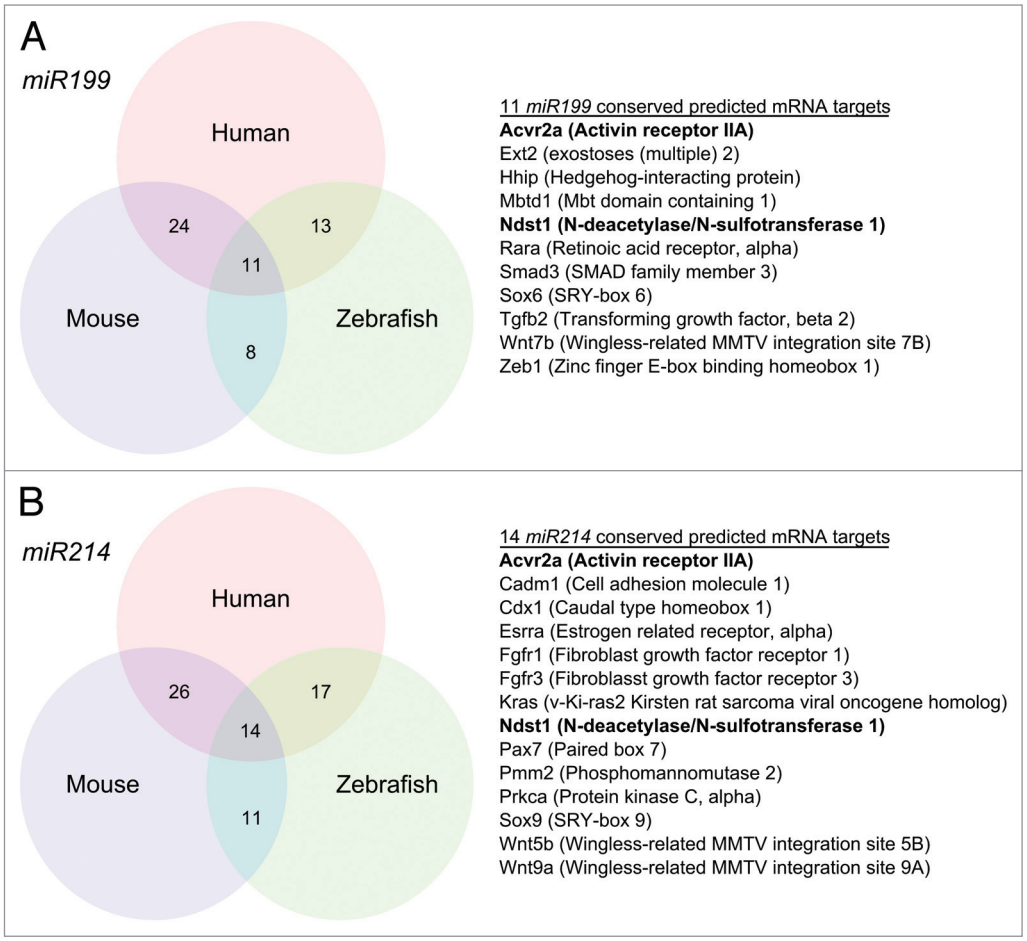

Figure 6. Venn diagrams showing the number of skeleton-related mRNA transcripts that are predicted targets for *miR199* (**A**) and *miR214* (**B**) and conserved between human, mouse, and zebrafish. The list of genes predicted to be targeted in all three species is given on the right side of the corresponding diagram.

about 300 nt in both directions from the miR gene and are gene specific for each miR because sequences surrounding the miR genes fail to align (**Table S2**). The *dnm3* probe spans the full transcript from the first to the last coding exon. We first performed whole mount in situ hybridization (WISH) on embryos from 24 hpf to 72 hpf (**Fig. 4**). Results showed that *dnm3* displayed a strikingly different expression pattern than *miR199-3* and *miR214*. Transcripts of *dnm3* were detected in the developing nervous system from 24 hpf to 72 hpf with predominant expression in the neural tube, brain and eyes (**Fig. 4A–D**). In contrast, *miR199-3* and *miR214* were expressed in different tissues than *dnm3* (**Fig. 4E–L**). Both miRs showed substantial expression in the mesenchyme surrounding developing craniofacial skeletal elements but no expression within the skeletal elements themselves, including the ceratobranchial arch at 24 hpf and the entire craniofacial skeleton at 48 and 72 hpf (**Fig. 4H and L**). In addition, and in contrast to *miR214*, *miR199-3* displayed strong expression in the notochord (**Fig. 4F and G**) while *miR214* showed weak expression in developing somites at 24 hpf (**Fig. 4I**) and weak expression in the developing brain throughout the period of development we studied (**Fig. 4I–K**). To confirm the cellular localization of *miR199-3* and *miR214* transcripts, we performed in situ hybridization on coronal and transverse cryosections of 72hpf zebrafish embryos (**Fig. 5**). Results agreed with expression patterns observed in WISH: both miRs displayed similar expression domains in the mesenchyme surrounding developing skeletal elements of the craniofacial skeleton. Interestingly, no miR expression could be observed within chondrocytes, the perichondrium or the epidermis.

We conclude that the host *dnm* gene and its *miR* guests are regulated differently. In contrast, the expression patterns of *miR199-3* and *mir214* are highly similar, as would be expected if they were transcribed as a unit, thus supporting the RT-PCR results. The differential expression of both miRs in some additional specific areas, however, such as *miR199-3* but not *miR214* in the notochord, suggests that the two genes are in some aspects regulated independently of one another.

Together, these experiments demonstrate that *miR199-3* and *miR214* are co-regulated in part through the expression of the common *pri-miR199-214* transcript. However, *miR199-3* and *miR214* display independent expression, which could be the result of independent regulation of expression; for example, from independent promoters, or differential degradation of one or the other miR of

the common transcript. Nevertheless, the most abundant expression of *miR199-3* and *miR214* around the developing skeletal elements strongly suggests an important function of both miRs in craniofacial skeletal development, and that their functions may be cooperative.

Discussion

An understanding of the evolutionary mechanisms that lead to the origin and diversification of miR gene families remains elusive. Unlike protein coding genes,[59] miR gene duplicates are commonly retained after genome duplication events, which leads to an increase in miR gene number over time.[9] Our analysis of *miR199-214 gene* cluster evolution provides an interesting case study for miR gene locus evolution. We found a tight evolutionary relationship between *Dynamin* genes and *miR199* and *miR214* genes: each *miR199* and *miR214* gene family member is located within orthologous introns of a *Dynamin* family gene. Because DNM-coding genes are located on opposite strands from the *miR199-214* genes, these miRs are not miRtrons (miRs whose pri-miRs correspond to a coding-gene spliced intron[60]), which is consistent with the different expression patterns observed in zebrafish for *Dnm3* and its *miR199-214* guests.

We also conclude that the *miR199-214* cluster is vertebrate-specific because it is absent from the genomes of non-vertebrate animals, including non-vertebrate chordates. This miR cluster thus provides an example of the dramatic expansion of miR gene number documented for the vertebrate radiation, which has been hypothesized to be related to increasing complexity of body plan organization.[8,9,10,61]

The conservation of *miR199* paralogs with host *Dynamin* paralogs generally supports the hypothesis that intronic-miR duplicates are likely to be retained along with their host gene after genome duplication. The *miR199-214* cluster, however, illustrates some exceptions to this generalization. The loss of a *miR199-1* paralog along with the *Dnm1* host after the VGD2 suggests that even when a miR gene and its protein coding host gene are independently regulated, they can both be lost. Also, we show that even if a duplicated *Dnm* gene is conserved throughout evolution, the intronic miR can independently be lost, as depicted by the loss of *miR199-2b* gene within the duplicated *dnm2b* gene in teleosts. This shows that the retention of miRs within coding genes is not always guaranteed in evolution subsequent to duplication events and that the miR sequence can be lost independently of the host gene. For the *Dnm*/*miR199-214* case, we did not find the retention of a duplicated intronic miR with the loss of its host coding-gene, but this type of retention has been described in medaka for a *miR499* duplicate in the *myh14* gene locus.[62] In zebrafish, an intergenic miR, *miR10*, ancestrally located within *hoxd* gene cluster, was retained after the teleost genome duplication despite the loss of all *hoxdb* protein coding genes.[12]

Another interesting feature of *miR199-214* gene cluster evolution is the lineage-specific loss of miR duplicates. For many vertebrates, conclusive evidence for the loss of miR paralogs is lacking because of genome assembly issues. But in a few cases—e.g., loss of *miR199-1* in *Xenopus* and the loss of some *miR199* genes in some teleosts—high-quality genome sequence evidence shows that different vertebrate lineages lost miR duplicates without the loss of the host coding-gene, providing a rare example of secondary loss of a miR during evolution.

In summary, among the four *Dnm* paralogs that emerged in vertebrates, one of them, the *Dnm1* VGD2 duplicate, was lost along with its *miR199* gene. Following the TGD, interestingly, all six *dnm* paralogs and miR genes were retained with the unique exception of the subsequent loss of the *miR199* paralog located within *dnm2b*.

Finally, the emergence of a new *miR* gene (*MIR3154*) within the mammalian *Dnm1* gene and two new miRs (*MIR638* and *MIR4748*) within the primate *DNM2* gene provides examples of novel miR gene family origin within vertebrate lineages.[63,64] Furthermore, the evolution of the complementary strand of *miR214* into a functional processed miR, *miR3120*, reveals a new type of miR emergence by complementarity with the mirrored miR gene. Altogether, the evolution of the *miR199-214* cluster provides various examples of duplication, retention, and lineage-specific loss or gain of miR sequences illustrating the range of processes in miR gene evolution.

Our study shows that the *miR199-214* cluster accumulated over time. We show that the most parsimonious evolutionary scenario is for the emergence of *miR199* before the first round of vertebrate whole genome duplication, and then *miR214* arose near *miR199-3* after the second round of vertebrate whole genome duplications. The sequential formation of the *miR199-214* cluster suggests that the functions of *miR199-3* and *miR214* may be cooperative because they became tightly associated in a cluster. Our RT-PCR experiments amplified a single primary transcript containing both *miR199* and *miR214*, demonstrating a common regulation of the cluster, as has been demonstrated both in mouse[25,27] and human.[28] Loebel et al. (2005) suggest that the *mir199-214* primary transcript is evolutionarily conserved within amniotes based on EST reads retrieved from genomic databases. None of those EST sequences, however, spans the distance between the two miRs. Because each EST matches only one or the other miR, EST data provide no evidence that *miR199* and *miR214* are expressed in a common transcript. In contrast, our RT-PCR experiments show by the analysis of in vivo transcription that these miRs come from a common transcript in zebrafish. The short distance between these miRs in vertebrates (1207 nt in the Japanese medaka, 2179 in zebrafish, 5445 in mouse, 5628 in human, and 5750 nt in chicken) makes a single transcript reasonable in all these species. Evolutionary conservation of sequence, position, and common expression of the cluster all suggest that *miR199* and *miR214* play an important common role in gene regulation in vertebrate development.

Our demonstration that *miR199-3* and *miR214* display the same dynamic pattern of expression reinforces the hypothesis that these two miRs may regulate a common function. Furthermore, the finding that all *miR199* genes except *miR199-2* produce the same mature sequence suggests identical or similar targets for these miRs, and hence, redundant functions during

zebrafish development. This situation raises the question: why should organisms retain multiple copies of genes encoding the same miR sequence that are expressed with the same expression pattern? The retention of miR duplicates producing the same mature sequence may reflect the importance of dose-dependent effects on targeted transcripts; such redundancy might confer developmental robustness and allow the fine-tuning of gene regulation. Redundancy of miRs within a family or a cluster has already been suggested in several cases such as the *miR17-92* cluster during development[65] and *miR30* family members in breast cancer.[66] Furthermore, the generation of numerous miR gene deletion mutations in *Caenorhabditis elegans* led to the conclusion that most miRs are not individually essential for development or viability, thus supporting the hypothesis that many miRs function redundantly.[67] Because *miR199-1a* and *miR199-2* show developmental expression patterns that differ from other *miR199* genes, sub-functionalization of regulatory features or the evolution of novel regulatory features (neofunctionalization) may have occurred in this case.[68] Also, because *miR199-2* has two nucleotide differences from other *miR199* genes in the mature *miR199-2-3p* strand (the major strand of all *miR199* genes[52]), *miR199-2* may interact with a different constellation of targets, and hence, have a different function. While pairing rules for miR repression functions may generally depend on perfect or nearly perfect Watson-Crick complementary pairing of the target to the miRNA seed,[5,6] other non-canonical pairings are possible.[69] For example, a G-bulge at position 6, called the "pivot," in about 15% of miR-target associations, has been functionally demonstrated,[70] as well as the unusual pairing of *miR214* on *Disp2*.[26] These situations highlight the challenge bioinformatic tools must overcome to efficiently predict miR:mRNA functional associations.

The developmental expression profile of the host gene *dnm3*, and its two miR guests were different. The *dnm3* gene is highly expressed in the nervous system,[25,27,55] but *miR199* and *miR214* are expressed strongly in mesenchyme and perichondrium around the developing craniofacial skeletal elements. Our results agree with prior observations in zebrafish[16,71] and mouse, where the common *miR199-214* transcript and mature miRs are expressed in perichondrial cells, periarticular chondrocytes, tracheal cartilage, limb mesenchyme, and most tissues in the upper and lower jaw.[25,27] The expression of these vertebrate-specific miRs occurs mainly in vertebrate-specific structures, such as the skeleton, suggesting a possible role in the evolutionary origin of vertebrate-specific features. A role of *miR199-214* in skeletogenesis is confirmed because *miR199-214* cluster-knockout mice display skeletal abnormalities, including craniofacial defects, neural arch and spinous process malformations, and osteopenia.[27]

A number of functional studies implicate *miR199* and *miR214* in skeletogenesis. Twist-1 is an important protein in skeleton formation[72,73] and Twist-1 regulates *miR199-214* cluster expression in mouse.[28] *MIR199* expression also responds to BMP2 induction in human cell lines and inhibits chondrogenesis by downregulating *SMAD1*,[29] a regulator of bone and cartilage formation and development.[74] Recently, *miR214* has been shown to inhibit bone formation in human cell lines both by targeting *ATF4*, a gene encoding one of the main transcription factors required for osteoblast function[31] and by suppressing osteogenic differentiation of C2C12 myoblast cells by targeting *SP7*, an osteoblast-specific transcription factor.[30,75]

To predict putative mRNA targets for *miR199* and *miR214*, we used miRZ[91] for mouse and human and TargetScanFish[92] for zebrafish. We then narrowed down the list by keeping only the genes having GO term associated with the skeleton in at least one of the three species.[93] Our analysis revealed 11 and 14 putative skeletal mRNA targets for *miR199* and *miR214*, respectively conserved among all three species, and many more putative mRNA targets conserved across only two of the three species (**Fig. 6**; **Fig. S6**). These predicted targets include several genes such as *Ext2*,[76,77] *Rara*,[78] *Fgfr1* and *Fgfr3*,[79] and *Sox9*.[80,81] No interaction between transcripts of these protein coding genes and *miR199* or *miR214* have been yet demonstrated in vivo, so this provides an important avenue for future research. Interestingly, among those predicted targets, two genes, *Acvr2a* and *Ndst1*, which play important roles in skeletogenesis[82,83], are predicted to be targets for both *miR199* and *miR214* in all three vertebrate species studied, suggesting a cooperative action of this miR cluster in the regulation of expression of those two protein coding genes. Furthermore, many other transcripts are shown to be targeted by both miRs but not conserved across all three species (**Fig. S6**). Together, the identification of evolutionary conserved targets and our results in zebrafish provide both tissue and temporal expression data consistent with a cooperative role of *miR199* and *miR214* in skeleton formation and morphogenesis that is conserved across vertebrate lineages. Furthermore, this role in skeletal development may be an important factor in bone formation or structural maintenance leading to pathologies, such as craniofacial birth defects, osteoporosis, or osteoarthritis.

Materials and Methods

Because *miR199* and *miR214* are located in *Dynamin* gene introns, we retrieved *Dynamin* gene sequences along with *miR199* and *miR214* precursor sequences from Ensembl,[84] NCBI, and miRBase (Release 20).[52] Sequences were gathered from the reference genomes of five sarcopterygian vertebrates (human *Homo sapiens* GRCh37, mouse *Mus musculus* GRCm38, chicken *Gallus gallus* Galgal4, frog *Xenopus tropicalis* JGI_4.2, and coelacanth *Latimeria chalumnae* LatCha1), eight teleost fish (zebrafish *Danio rerio* Zv9, Atlantic cod *Gadus morhua* gadMor1, Japanese pufferfish *Takifugu rubripes* FUGU4, Japanese medaka *Oryzias latipes* MEDAKA1, three-spined stickleback *Gasterosteus aculeatus* BROADS1, green spotted pufferfish *Tetraodon nigroviridis* TETRAODON8, tilapia *Oreochromis niloticus* Orenil1.0, and platyfish *Xiphophorus maculatus* Xipmac4.4.2), and the spotted gar *Lepisosteus oculatus* LepOcu1, an outgroup to the teleosts that diverged before the Teleost Genome Duplication (TGD).[85] We searched for *Dynamin*, *miR199*, and *miR214* sequences in all Ensembl

available invertebrate reference genomes, but could find no trace of *miR199* or *miR214* sequences and only one *Dynamin* gene in each species. Among *Dnm* and *miR* sequences reported here (**Table S1**), all were predicted in available Ensembl genome assemblies at the time of submission, but only some had a miR name annotation (**Table 1**). The only sequences that were annotated de novo during this study are those from the recently sequenced spotted gar *Lepisosteus oculatus*. **Table S1** gives the accession numbers and genomic locations of sequences used in this study.

To understand phylogenetic relationships among miR homologs, we compared the genomic location of *miR199* and *miR214* genes in a phylogenetic context. Genomic analysis for conserved sequences was performed with zPicture[86] by aligning the zebrafish *dnm3* gene intron containing the *miR199-214* cluster to orthologous exons in other vertebrates. Fasta alignments were done using BioEdit, and secondary structure visualizations were edited with VARNA v3.9[87] based on Stockholm alignment and consensus secondary structures predicted by CMfinder 2.0.[88] Synteny conservation analysis of the *Dynamin* loci was performed using the Synteny Database.[51] Evolutionary trees for Dynamin proteins were constructed using PhyML through the Phylogeny.fr web server with Gblocks set up for more stringent selection and the minimum of SH and Chi-2-based methods with the WAG substitution model.[89] We used Dynamin protein sequences from only species in which a full set of *Dynamin* genes could be found: three tetrapods (*Homo sapiens*, *Mus musculus*, and *Xenopus tropicalis*), two teleost fish (*Danio rerio* and *Oreochromis niloticus*), and the teleost outgroup Spotted gar *Lepisosteus oculatus*. The tunicate urochordate *Ciona intestinalis* served as a non-vertebrate chordate outgroup. The phylogenetic tree was rooted using human and *Ciona* Dynamin1-like protein (Dnm1-L), which is the most closely related protein to the Dynamin family.[90]

Targets for miRs were predicted using miRZ[91] for mouse and human and TargetScanFish[92] for zebrafish because of the coverage characteristics of these databases. Among predicted targets, only those having a GO term associated with skeleton[93] in at least one species were conserved. Skeleton-associated predicted targets were then cross-compared between the three species and analyzed for conservation.

To assess miR expression, we designed RT-PCR primers and optimized PCR protocols to specifically amplify each *pri-miR199* paralog. RNA was extracted from three separate clutches of zebrafish embryos using TRI Reagent following the manufacturer's instructions. RNA extracts were treated for DNA contamination using the DNA-free RNA kit (Zymo Research) prior to retro-transcription at 55 °C using $OligoDT_{20}$ primers. cDNAs were treated with RNaseH before PCR amplification of the target transcript. β-actin (ENSDARG00000037746) was used as a control to assess genomic DNA contamination. **Table S2** gives primer sequences, annealing temperatures, and amplicon sizes. In situ hybridizations were performed as previously described[58] using probes directed against the primary transcripts of zebrafish *miR199-3* or *miR214* (**Table S2**). Photographs of whole-mount in situ hybridization results and in situ hybridizations to histological sections were taken using a Leica M165FC stereomicroscope and a Leica DMLB microscope, respectively. All animal work was performed according to the University of Oregon IACUC approved protocol (#09–1BRRA).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: http://www.landesbioscience.com/journals/rnabiology/article/28141/

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